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SCREENING OF IMMUNOENHANCING DRUGS WITH ANTIVIRAL
ACTIVITY AGAINST MEMBERS OF THE ARENA-, ALPHA-,
AND ADENOVIRIDAE

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) Twenty-eight compounds or combinations of compounds were screened for their in vivo antiviral activity. Several preparations were found to be able to protect mice from challenge with mouse adenovirus but not Venezuelan equine encephalitis (VEE) virus. Only two compounds (AVS-1761 and AVS-1968) had any effect on survival of mice infected with VEE. Additionally, AVS-1758 was able to reduce the level of circulating VEE but was not able to alter the outcome of the infection. Macrophage activation for cytolysis of virally infected targets was found to be differ- ent from cytolysis of a standard tumor target in sensitivity to stimuli, kinetics of the response, and sensitivity to inhibitors. This indicated that detection of changes in effector functions in nonviral systems may not be applicable to viral systems. The ability of various potential immunomodulators to effect macrophage, natural killer cell, and cytotoxic T-cell responses were measured using virally infected cells as targets. (continued on reverse side)						
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19. Finally attempts were made to develop a mouse-lethal strain of Pichinde virus. Two plaque-type variant that grew well on mouse L-929 cells were isolated. However, neither produced disease in adult mice.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985.

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LIST OF ABBREVIATIONS

DMEM	Dulbeco's modified Eagles' medium
FBS	Fetal bovine serum
HMEM	Hepes buffered modified Eagles' minimal essentials medium
IFN	Interferon: γ gamma: α alpha: β beta
LAL	Limulus amoebocyte lysate
LPS	Lipopolysaccharide
MAdv	Mouse adenovirus
rTNF	recombinant tumor necrosis factor alpha
TLCK	N-p-tosyl-L-lysine chloromethyl ketone
TNF	Tumor necrosis factor alpha
VEE	Venezuelan equine encephalitis virus
VSV	Vesicular stomatitis virus

INTRODUCTION

This is the final report of a contract to screen immunomodulatory drugs for antiviral activity against a variety of viruses. Our original plan of attack had three levels of investigation. The first was to determine which drugs caused an increase in resistance to lethal viral infections. The concept was to then concentrate on only those drugs that had demonstrable survival benefit with more than one virus. These drugs would offer the best candidates for agents to increase resistance to viral infections where the exact nature of the viral agent was unknown. The second level of screening was to be an evaluation of the various compartments of the immune response in the hope that the effective drugs might share the ability to modulate a particular segment of the immune response. The third level of screening was designed to measure the changes in the specific antiviral response in the presence of an active viral infection.

MATERIALS AND METHODS

Endotoxin Detection.

All reagents were tested for the presence of bacterial endotoxin by the gel formation, *Lymulus* amebocyte lysate (LAL) test (1). The LAL was obtained from Associates of Cape Cod (Woods Hole, MA) with a 0.03 endotoxin unit per ml sensitivity. The sensitivity was confirmed with each test by the titration of standard lipopolysaccharide (LPS). Materials that test negative in this test were defined as endotoxin free.

Cells and Cell Culture.

Modified Eagles' minimal essential medium (HMEM) Ham's F12 and Dulbecco's modified Eagles' medium (DMEM) were prepared from a powdered mix (HyClone Laboratories, Logan, UT) and supplemented with glutamine (2 mM, Flow Laboratories, McClean VA), sodium bicarbonate (2 mg/ml, Sigma Chemical Co., St. Louis, MO), and HEPES (15 mM, Research Organics, Cleveland, OH). All serum, including fetal bovine serum (FBS) was obtained from HyClone Laboratories. Clone 929 of L-cells (CCL 1) were obtained from the ATCC, Rockville, Maryland, and were propagated in HMEM + 10% FBS. Vero (African green monkey kidney) BALB/c3T3 cells were obtained from Dr. Gillespie (University of North Carolina Cancer Center, Chapel Hill, NC) and propagated in HMEM + 2% FBS or a 1:1 mixture of F12 and DMEM with 10% FBS, respectively. Chicken embryo cell (CEC) were produced by trypsinization of 9-11 day old chicken embryos according to the method of Scherer (2) except that HMEM + 10% FBS or HMEM + 4% chicken serum were used as growth media. The preparation of bone marrow culture derived macrophages has been previously described (3).

Viruses.

Venezuelan equine encephalitis (VEE) strain 68U201 (4) was obtained from Dr. Peter Jahrling, USAMRIID, and was propagated in primary chicken embryo cell culture to prepare a working stock. Pichinde strain An3739 (5) was obtained from the same source and was propagated in Vero cell cultures to prepare a working stock of virus. Pichinde strain An4763 (6) was obtained from the Southern Research Institute and propagated in Vero cells. Vesicular stomatitis virus (VSV) was propagated in L-929 cells. A lung tropic, plaque purified strain of mouse adenovirus (MAdV) was derived by one of the investigators (ALW) and has been previously described (7). Working stocks of MAdV were prepared in roller cultures of L-929 cells.

Mixed cell bed cultures were prepared by the addition of 5 ml of HMEM supplemented with 5% heat inactivated calf serum containing 9×10^5 Vero and 9×10^5 L-929 cells to a 60 mm tissue culture dish. The dish was incubated overnight and then overlaying medium removed and the cells infected by the addition of 0.5 ml of an appropriate 10-fold dilution of Pichinde virus or plaque progeny. After 1 hour for adsorption, the cultures were overlayed with 8 ml of HMEM supplemented with 5% heat inactivated calf serum and 1%

agarose. A neutral red containing overlay medium was added on day 4 and plaques were observed and or picked on days 5 or 7. Plaques progeny were isolated by the removal of an agarose plug from the overlaying medium directly over the center of the desired plaque using a sterile pasteur pipet cut to give an outside diameter of 2.5 mm. The agar plug was emulsified in 1.0 ml of medium. The resultant plaque progeny were then further purified by plaquing 10-fold serial dilutions on duplicate cultures of the appropriate cell type and reselecting plaques from dishes developing only a few, well-isolated plaques.

In Vivo Tests.

A compound was administered as recommended by the source and then the mice were challenged with either 400 pfu of the 68U201 strain of VEE, subcutaneously or $>2 \times 10^7$ pfu of MAdV, intraperitoneally. Mice were observed daily for deaths for 31 days. Alternatively, groups of mice were treated with immunomodulators, challenged with VEE, as described, and then bled from the retroorbital sinus on days 1 and 2. On day 4 after infection, mice were sacrificed and a 10% (w/v) suspension made from their brains. All samples were stored at -80° until tested for virus content by titration on CEC cultures.

Cytotoxicity Assays.

Compounds were tested for their ability to modify the cytotoxic T cell response generated due to the injection of either the TC-83 strain of Venezuelan equine encephalitis (VEE) virus or to the histoincompatible cell line P815. Groups of C3H/HeN (H-2^k) mice were treated with a compound or with a placebo and then challenged with either subcutaneously with 10^4 pfu of VEE strain TC-83 grown in chicken serum or intraperitoneally with 10^6 P815 (H-2^d) cells, or not injected with an antigen. Eleven days later, mice were sacrificed and a single cell suspension prepared from their spleens. The spleen cells from all mice in a group were pooled, and the pool tested at various effector to target ratios against four different ⁵¹-Chromium labelled target cells. The target cells were VEE-infected L929 cells (histocompatible, virus specific), Vesicular stomatitis virus (VSV) infected L929 cells (histocompatible, virus nonspecific), P815 cells (allogeneic challenge), and VEE infected BALB/c3T3 cells (histoincompatible, virus specific). BALB/c3T3 (3T3) cells are of the same H-2 type as P815 and these two cells served as mutual internal controls. Further, changes in natural killer cell (NK) activity could be addressed by comparing the results obtained from spleen cells obtained from control and treated animals and tested with L929-VSV cells or 3T3-VEE cells. P815 cells are relatively NK resistant. The data from all ⁵¹Cr-release assays were expressed as specific release based on the formula: percent specific release = $(\text{experimental} - \text{spontaneous}) / (\text{maximum} - \text{spontaneous}) \times 100$.

Macrophage activation assays were performed as previously described (8) except that virus-infected BALB/c3T3 cells were used as targets. These cells were used because the cells are histoincompatible with the C3H/HeN macrophages used. Further, uninfected BALB/c3T3 cells are seen as normal by macrophages and not lysed, while either transformed or infected cells make satisfactory targets. Bone marrow culture-derived macrophage monolayers, prepared in 96-well plates were treated with 0.1 ml of various stimuli, as described in the text, for either 4 or 24 hrs. Each stimuli was tested in triplicate. The stimuli were then removed and the monolayers washed twice with warm H-MEM + 10% FBS. Targets were added to yield 2×10^4 ⁵¹Cr-labelled targets in a total volume of 0.2 ml in each well. The plates were incubated at 37° in a moist 5% CO₂ in air atmosphere for sixteen hr. The uppermost 0.1 ml of medium was removed from each well and the amount of released radioactivity determined in an automatic gamma spectrometer.

Targets used were: P815, BALB/c3T3, Vero, an African green monkey kidney cell, vesicular stomatitis virus (VSV) infected BALB/c3T3 (3T3-VSV), VSV-infected Vero (Vero-VSV), and Venezuelan equine encephalitis virus, strain TC-83, (VEE) infected BALB/c3T3 (3T3-VEE). P815 cells were suspended into the overlaying medium and counted.

Uninfected BALB/c and Vero cells were prepared by trypsinization of near confluent monolayers and washed by centrifugation in H-MEM + 10% FBS. Near confluent monolayers of either BALB/c3T3 or Vero cells were infected with a multiplicity of infection (MOI) of 3. After 45 min at 37° for virus adsorption, monolayers were washed once with H-MEM + 10% FBS and once with H-MEM to remove unadsorbed virus. The cells were then removed from the flask by trypsinization and washed by centrifugation with H-MEM + 10% FBS. All cells were labelled by exposing 1.2×10^7 cells to 0.275 mCi of ^{51}Cr as sodium chromate (Amersham, Arlington Heights, IL) in a total volume of 1.0 ml for 80 minutes. Cells were assessed for viability by trypan blue exclusion just before addition to the assay plate and were >95% viable.

Bacterial lipopolysaccharide (LPS) was phenol extracted and purified (lipid A rich, fraction II) [18] from *Escherichia coli* O111:B4 and was the generous gift of Dr. D. C. Morrison (Kansas University Medical Center, Kansas City, KS). Gamma interferon (γ -IFN) was recombinant rat gamma interferon (Amgen Biological, Thousand Oaks, CA). Alpha (α -IFN) and beta (β -IFN) interferons were purchased from Lee Biomolecular, San Diego, CA (#22061, Lot 83002, 2.7×10^6 IRU/mg; #20181, Lot 83055, 1.8×10^6 IRU/mg, respectively). A mixture containing both α -IFN and β -IFN (α/β -IFN) was prepared by mixing equal activities of each purified interferon. This mixture was used for most experiments because preliminary experiments showed little difference in activity between α -IFN and β -IFN, which was in agreement with previous reports (9). Interferon (IFN) preparations were assayed for antiviral activity by plaque reduction of VSV on monolayers of L-929 cells (9,10). Each dilution was tested in triplicate and activity was expressed in International Reference Units (IRU), with mouse fibroblast interferon as a reference standard (NIH). Anti-mouse IFN (Alpha and Beta, 1×10^4 IRU/ml) globulin in phosphate buffered saline was lot # 84005 of catalog # 21031 from Lee Biomolecular.

Inhibition studies were conducted in a similar manner except that targets were added in medium containing the inhibitors.

Tumor Necrosis Factor mRNA Production

The production of Tumor necrosis factor alpha (TNF) mRNA by bone marrow culture derived macrophages was determined by probing with a plasmid containing a 1700 bp fragment of TNF cloned into the polylinker of PUC 9 and obtained from Dr. Steven Taffet, SUNY Upstate Medical Center, Syracuse, NY. The probe was labelled with ^{32}P dCTP (Amersham, Arlington Heights, IL) by nick translation using a commercial kit (BRL, Gaithersburg, MD) and detected by radioautography.

Macrophages (1×10^7) were exposed to various stimulatory conditions, as described in the text, and then chilled, scrapped from the dish and pelleted. The cells were lysed with NP-40 and the nuclei pelleted. The RNA was then extracted from the supernatant and blotted onto nitrocellulose filter for probing by previously described methods (11). The intensities of the dots were determined by optical density scanning of the dried films.

Target Sensitivity to TNF

Recombinant murine TNF- α (rTNF) was obtained from Genzyme (Boston, MA). Chromium-labelled target cells (1×10^4) were exposed to increasing concentrations of rTNF in a total volume of 200 μ l HMEM + 10% FBS for 16h. Kill was measured by determining the amount of specific chromium release using the formula given under cytotoxic assays. The ability of anti-TNF antibody (Genzyme) to block cytotoxicity was determined using a standard ^{51}Cr -release assay described above, except that the targets were added in the presence of the anti-TNF antibody.

RESULTS AND DISCUSSION

Twenty-eight compounds or combination of compounds were screened at various doses to determine if there were any with in vivo antiviral activity. These results have been reported previously (12,13,14). They are summarized in a qualitative manner in Table 1. Initially screening was done with both VEE and MAdV. It became obvious that many compounds were able to modulate the outcome of infections with MAdV but not VEE. Therefore, under the direction of the CTO, subsequent compounds were tested with only VEE. The original intent was to include Pichinde in this first screening. However, the amount of material available was not sufficient for use in the guinea pig model. Only two compound had any effect on the survival of mice infected with VEE (AVS-1761 and AVS-1968).

Since many of the immunomodulators had an effect on survival after MAdV but not VEE challenge, we tried to determine if some of the immunomodulators might be having an effect on the course of VEE infection

that was not detectable using death as an endpoint. Therefore, we determined if there was an effect on the level of virus either in the blood or brain after infection. The results are summarized in Table 2 and have been previously reported (13). The two drug that had a positive effect on survival were also able to limit the amount of virus in the whole animal. In addition, AVS-1758 was able to cause a reduction in circulating virus as compared to controls but was unable to significantly alter the outcome of the infection. AVS-2776, AVS-2777, and AVS-2778 did not result in either increased survival or decreased circulating virus.

Level 2 screening was meant to use standard assays to evaluate immunomodulatory effects of the drugs. A combination of results from early level 2 testing (12) and other work in the laboratory (14,15) indicated that this might not be the best way to proceed. Specifically, when macrophage activation assays were run using a standard tumor target (P815) and a virally infected nontumorigenic target (BALB/c3T3), we obtained different results in terms of sensitivity to activators and the kinetics of the response (15). Further, lysis of P815 targets was more sensitive to inhibition by N-p-tosyl-L-lysine chloromethyl ketone (TLCK) than was lysis of virally infected BALB/c3T3 targets (14). On the other hand, lysis of virally infected BALB/c3T3 targets could be inhibited by conducting the assay in glucose free medium the lysis of P815 could not be inhibited in that manner (14). Thus the results could not be attributed to one target being simply more fragile than the other. Rather, each macrophage-target interaction caused the expression of different cytolytic mechanisms or the targets had differential susceptibility to different cytolytic mechanisms that were expressed at the same level independent of the target involved.

Supporting the latter contention, it was found that the production of TNF mRNA in response to LPS paralleled the expression of cytolytic activity for virally infected cells but not the P815 tumor target both in dose response and time course of expression (14). The amount of TNF mRNA was not effected by the presence or absence of either target cell. Further, P815 and uninfected BALB/c3T3 cells were resistant to lysis by rTNF. However, BALB/c3T3 cells infected with either VSV or VEE became susceptible to lysis by rTNF. 3T3-VSV were more sensitive to lysis by rTNF than were 3T3-VEE. This represented the same relative sensitivity to lysis of these targets in the presence of LPS-activated macrophages. Addition of anti-TNF antibody to the macrophage target system was able to suppress cytolytic activity for virally infected cells but not for P815 (14). Thus, at least in the case of TNF, the difference in the phenotypic differences in the interaction of the macrophage and the different targets results in the differential susceptibility of each target to the TNF produced at the same level by the macrophage in both systems.

Beyond the specific point of virally infected target being sensitive to TNF these data underline an important concept. Changes in effector functions measured in a nonviral system may not be relevant to effector functions measured in a viral system.

The effects of immunomodulators on macrophage activation were measured using virus infected BALB/c3T3 cells as targets. Two modes of exposure were used. The mice were treated with drug as in the survival experiments and the macrophages harvested when virus would have been administered. The second mode of exposure was the inclusion of the drug in the culture medium of bone marrow culture derived macrophages for 24 h before assay. In both cases it was important to use only drugs that could be prepared in an endotoxin free state. Macrophages are extremely sensitive to very low levels of endotoxin (15). Table 3 shows the results of endotoxin assays using a concentration derived from the dose of each drug administered in vivo. Only the LAL negative drugs were used for the macrophage assays.

Table 4 shows a sample of the type of data obtained from an assay with AVS-1018 and in vitro exposure of the macrophages to the drug. Such exposure rendered the macrophages more responsive to activation for cytolysis of VEE infected BALB/c3T3 cells than control macrophages. Macrophages exposed to gamma interferon or γ IFN plus LPS did not show any change in reactivity compared to controls. Data from the other drugs is summarized in Tables 5 and 6. In both in vivo and in vitro assay systems AVS-2776 showed the greatest ability to increase macrophage responsiveness to activating stimuli. AVS-1968 was actually inhibitory at least in the in vitro tests. These data indicate that increasing macrophage responsiveness to activating stimuli may not be advantageous for the survival of the animal with VEE infections since AVS-1968 was one of only two drugs that enhanced survival while AVS-2776 had no effect on survival.

Due to the differences in the results for macrophage activation using a tumor target or a virally infected target when we looked at cytolytic T-lymphocyte (CTL) activity, we used both a standard anti-allogeneic system and measurement of virus specific CTL activity induced during a natural viral infection. Figures 1A-D show that we were able to detect virus specific CTL activity and that one drug (AVS 2149) was able to increase the level of activity. AVS-2149 also cause a decrease in the anti-allogeneic response. The data from other drugs is summarized in Table 7. Both AVS-1761 and AVS-1968 that increased survival in the face of VEE infection also showed a decrease in anti-viral CTL activity. This decrease may result because the drugs result in low levels of circulating virus (13) by some as yet to be determined mechanism and this in turn results in lower levels of antigen available for stimulation of the CTL response. It is also apparent that the results from the anti-allogeneic and anti-viral systems do not always yield the same results.

The final area of research under this contract was a series of attempts to generate a mouse adapted Pichinde strain that could be used as a mouse model for Arenavirus infections. High titered Pichinde strain AnCo 3738 was inoculated into mice and attempts were made to recover virus from the spleen and liver at daily intervals after inoculation. Little or no virus was recovered from these organs indicating a failure of the virus to replicate. A second strategy was to infect mixed bed composed of permissive Vero cells and mouse L-929 cells. We then picked plaques that showed complete lysis indicating that the virus had cause cytopathic effects in the mouse cells. After several such passages a strain was isolated that grew to high titer in L-929 cultures. In the process of titering this virus, it was noticed that the plaques were heterogeneous. Two plaque type variants were isolated. One showed only minimal and transient cytopathology on L-929 cells while the other was completely cytolytic. To determine if this was a widespread occurrence, we attempted to isolate similar plaque type variants from another Pichinde strain (Co 4763). Again, two plaque type variants with similar characteristics were isolated. Unfortunately, none of the isolates were able to cause disease in adult mice.

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Table 1. Survival of immunomodulator treated mice after lethal challenge with VEE or MAdV. Groups of 5 mice each were treated with immunomodulator or control as previously described (12,13,14) and then challenged with 400 pfu of 68U201 strain of VEE sc or $>2 \times 10^7$ pfu of strain pt4 of MAdV ip. Mice were observed for 28 days and compared to similarly infected control treated mice. Results are summarized by "+" better than control survival; "0" no improved survival, and "nt" not tested.

DRUG	VEE	MAd
BPV	0	+
EP-LPS	0	+
L121 + T150R1	0	+
L121 + T150R1 + EP-LPS	0	+
L121 + T150R1 + AVS-1968 (low)	0	nt
F68 + AVS-1968	+	nt
T150R1	nt	+
T1501	nt	+
L121	nt	-
T110R1	nt	+
TNF-alpha	0	+ / 0
IL-2	0	nt
AVS-1758	0	-
AVS-1761	+	nt
AVS-1968	+	nt
AVS-2149	0	nt
AVS-2150	0	nt
AVS-2153	0	nt
AVS-2154	0	nt
AVS-2155	0	nt
AVS-2156	0	nt
AVS-2157	0	nt
AVS-2158	0	nt
AVS-2776	0	+
AVS-2777	0	+ / 0
AVS-2778	0	0

Table 2. Detailed Level 1 screening for in vivo antiviral activity of immunomodulators against Venezuelan equine encephalitis virus challenge. C3H/HeN mice were treated with immunomodulators by route, time, and dose indicated. Time is indicated in days before (-) or after (+) virus challenge. Mice were then challenged with a subcutaneous injection containing 400 pfu of 68U201 strain of Venezuelan equine encephalitis. Mice were observed daily for deaths.

Compound	Dose mg/kg	Route	Time days	# Dead / # Infected	MTD	MST
BPV	20	ip	-7	10/10	6.8	7.0
diluent		ip	-7	10/10	7.1	7.5
EP-LPS	4	ip	-7	10/10	8.0	8.5
saline		ip	-7	10/10	6.9	7.5
IL-2	4 x 10 ⁶ U	subcut.	-1	10/10	8.5	9.0
saline			-1	9/9	8.2	9.0
L121:T150R1	100	ip	-7	10/10	6.7	6.5
L121:T150R1 +	100	ip	-7	10/10	5.5	5.5
EP-LPS	0.4					
L121:T150R1 +	100	ip	-7	10/10	5.7	6.0
AVS-1968	100	oral				
saline		ip	-7	10/10	6.8	6.5
L121:T150R1	100	ip	-7	10/10	7.2	7.5
L121:T150R1 +	100	ip	-7	10/10	7.2	7.5
AVS-1968	100	oral	-1			
saline		ip	-7	10/10	7.8	8.0
AVS-1758	1.2	subcut.	-1	10/10	8.3	8.0
saline		subcut.	-1	5/6	8.0	9.0
AVS-1758	4	ip	-.25	10/10	8.8	9.0
saline		ip	-.25	10/10	7.1	8.0
AVS-1761	0.4	ip	-1	10/10	11.4	11.0
saline		ip	-1	10/10	8.8	10.0
AVS-1761	3x0.4	ip	-.25.+3.+6	10/10	10.0	10.0
Saline		ip	-.25.+3.+6	10/10	8.5	10.0
AVS-1761	3x0.4	ip	-.25.+3.+6	10/10	13.3	12.0
saline		ip	-.25.+3.+6	10/10	7.9	8.0
AVS-1968	100	oral	-1	10/10	6.7	9.0
AVS-1968	2x100	oral	-3,-1	10/10	9.7	10.5
AVS-1968	2x100	oral	-1,+3	8/10	11.5	9.5
no treatment				10/10	9.0	9.0
AVS-1968	400	oral	-1	10/10	15.4	17
saline		oral	-1	10/10	7.3	8.0
AVS-1968	400	oral	-1	8/10	15.8	16
saline		oral	-1	10/10	8.5	8.0
AVS-2149	4.0	subcut.	-1	11/11	8.8	8.0
AVS-2149	40.0	subcut.	-1	5/5	8.0	7.0
saline		subcut	-1	11/11	8.0	8.0

AVS-2150	2/1	ip	-7	10/10	8.8	8.0
AVS-2150	4/2	ip	-7	10/10	8.9	8.0
AVS-2150	10/4	ip	-7	10/10	8.5	9.0
AVS-2154	1/2/1	ip	-7	10/10	7.2	7.0
AVS-2154	2/4/2	ip	-7	10/10	8.0	8.0
AVS-2154	4/4/4	ip	-7	10/10	7.9	8.0
saline		ip	-7	10/10	7.7	8.0
AVS-2153	3x4	ip	-3.0.+3	15/15	7.5	8.0
AVS-2154	3x4	ip	-3.0.+3	15/15	7.3	7.0
saline		ip	-3.0.+3	15/15	8.0	8.0
AVS-2155	4	ip	-7	9/9	9.6	10.0
AVS-2155	10	ip	-7	10/10	7.5	7.0
AVS-2155	20	ip	-7	10/10	7.4	7.0
AVS-2156	1	ip	-7	10/10	10.0	10.0
AVS-2156	2	ip	-7	10/10	9.1	9.0
AVS-2156	4	ip	-7	10/10	8.9	9.0
saline		ip	-7	10/10	9.3	9.0
AVS-2157	4	ip	-7	9/9	7.5	7.5
AVS-2157	16	ip	-7	10/10	7.6	8.0
AVS-2157	48	ip	-7	10/10	7.2	7.0
saline		ip	-7	10/10	7.2	7.0
AVS-2158	4	ip	-7	10/10	8.2	8.0
AVS-2158	20	ip	-7	9/9	6.7	7.0
AVS-2158	60	ip	-7	10/10	8.0	7.0
saline		ip	-7	9/9	8.4	9.0
AVS-2776	62	ip	-1	10/10	7.8	7.5
AVS-2777	62	ip	-1	10/10	8.6	8.5
AVS-2778	62	ip	-1	10/10	8.5	8.5
saline		ip	-1	6/6	8.0	9.0
AVS-2776	4x63	ip	-3.-1.1.3	9/9	8.6	9.0
methyl cellulose		ip	-3.-1.1.3	11/11	8.5	8.0
AVS-2776	125	ip	-.25	10/10	6.9	7.0
AVS-2777	125	ip	-.25	10/10	6.5	7.0
AVS-2778	125	ip	-.25	10/10	6.0	6.5
saline		ip	-.25	10/10	7.1	8.0

Table 3. Detailed Level 1 screening for in vivo antiviral activity of immunomodulators against mouse adenovirus strain pt4 challenge. C3H/HeN mice were treated with immunomodulators at doses and times indicated ant then challenge with an intraperitoneal injection of $>2 \times 10^7$ pfu of mouse adenovirus, strain pt4. Time is indicated as days before (-) or after (+) virus challenge. Mice were observed daily for death.

Compound	Dose mg/kg	Route	Time days	# Dead / # Infected	MTD	MST
EP-LPS	2	ip	-3	0/5	>21	>21
EP-LPS	2	ip	-2	4/10	16.5	21
EP-LPS	2	ip	-1	8/9	9.7	6.0
uninjected				5/5	4.0	4.0
L121 + T150R1	50	ip	-7	9/9	4.0	4.0
Control		ip	-7	10/10	4.0	4.0
L121 + T150R1	50	ip	-7	3/10	15.7	21.0
Control		ip	-7	10/10	4.4	4.0
L121 + T150R1	50	ip	-7	3/10	16.8	21.0
Control		ip	-7	10/10	4.2	4.0
T150R1	50	ip	-7	9/10	7.3	4.0
L122	50	ip	-7	8/10	3.7	4.0
T110R1	50	ip	-7	3/4	4.5	11.0
T1501	50	ip	-7	2/9	19.2	21.0
Saline		ip	-7	9/10	5.7	4.0
L121 + T150R1	50	ip	-7	1/10	19.5	21.0
T150R1	50	ip	-7	1/10	19.5	21.0
L121	50	ip	-7	5/9	11.6	5.0
Saline		ip	-7	7/8	6.1	4.0
L121 + T150R1	50	ip	-7	0/10	21.0	21.0
T150R1	50	ip	-7	4/10	14.7	21.0
L121	50	ip	-7	7/7	4.4	4.0
Saline		ip	-7	7/7	4.0	4.0
AVS-2776	100	ip	-1	8/10	10.5	3.5
AVS-2777	100	ip	-1	10/10	7.0	6.0
AVS-2778	100	ip	-1	10/10	4.5	4.0
Saline		ip	-1	9/10	6.0	4.0
AVS-1758	1.2	ip	-1	10/10	3.4	3.0
Saline		-1	9/10	6.0	4.0	
AVS-2149	4	ip	-.25	10/10	3.8	4.0
AVS-2149	4	ip	-1	10/10	4.1	4.0
Saline		ip	-.25	10/10	4.0	4.0

Table 4 . Inhibition of viral replication in vivo by immunomodulators. Groups of five mice were treated with immunomodulators by dose, route, and time indicated. Time is indicated in days before (-) or after (+) challenge with 400 pfu of 68U201 strain of VEE by subcutaneous inoculation. Individual mice were earpunched for identification and were bled from the retroorbital sinus 24 and 48 hours after challenge. Four days after challenge, mice were sacrificed, their brains removed and a 10% (w/v) suspension was prepared. After clarification by centrifugation, samples were filter sterilized. All samples were frozen at -75 until titered on chicken embryo primary cell monolayers. All values are expressed as the mean \pm standard error. NOTES: Values for mice in groups footnoted above were (a) 6.17, 6.17, 6.17, 6.28, 6.48; (b) 4.9, 6.17, 6.17, 6.17, 6.17; (c) 7.36, 6.17, 6.17, 5.79, 4.11; (d) 5.95, 6.17, 6.18, 6.17, 6.17.

Treatment	Dose mg/kg	Route	Time	Virus Titer Log ₁₀ pfu/ml or pfu/g		
				24 hr Blood	48 hr Blood	Day 4 Brain
Expt 1						
Saline		ip	-1	6.88 +/- .11	5.74 +/- .138	7.30 +/- .10
AVS-1968	100	oral	-1	5.05 +/- .45	5.99 +/- .122	7.19 +/- .109
AVS-1758	5	ip	-1.25	6.07 +/- .25	4.83 +/- .100	7.67 +/- .130
AVS-2776	250	ip	-1.25	6.63 +/- .20	5.79 +/- .108	8.06 +/- .107
AVS-2777	250	ip	-1.25	7.20 +/- .14	5.67 +/- .107	8.06 +/- .124
AVS-2778	250	ip	-1.25	7.02 +/- .15	5.36 +/- .120	7.90 +/- .112
Expt 2						
PBS		ip	-3.0-1	7.75 +/- .11	5.97 +/- .128	7.61 +/- .117
AVS-1968	100	ip	-3.0-1	6.14 +/- 0	5.29 +/- .144	6.43 +/- .150
Expt 3						
PBS		oral	-1	6.34 +/- .124	5.22 +/- .107	7.35 +/- .113
AVS-1968	400	oral	-1	6.17 +/- 0	5.3 +/- .114	4.13 +/- .125
Expt 4						
PBS		ip	-1.25,+3	6.98 +/- .103	5.11 +/- .103	7.99 +/- .104
AVS-1761	.4x2	ip	-1.25,-3	6.17 +/- 0	6.17 +/- 0	6.64 +/- .196
Expt 5						
PBS		ip	-7	6.41 +/- .119	6.63 +/- .122	ND
T150R1 + L12150		ip	-7	6.48 +/- .134	6.36 +/- .115	NE
Expt 6						
PBS		ip	-3.0,+3	6.65 +/- .127	5.54 +/- .108	7.59 +/- .118
AVS-2153	4x3	ip	-3.0,+3	6.32 +/- .119	5.06 +/- .106	7.74 +/- .116
AVS-2154	4x3	ip	-3.0,+3	6.21 +/- .129	5.10 +/- .113	7.53 +/- .114

Table 5. Comparison on the ability of immunomodulators to effect the survival of and viremia levels in mice challenged with VEE. Summary of data from Tables 2-4.

DRUG	VIREMIA	SURVIVAL
AVS-1758	+	0
AVS-1761	+	+
AVS-1968	+	+
AVS-2776	0	0
AVS-2777	0	0
AVS-2778	0	0

Table 6. Endotoxin content of immunomodulatory drugs. Compounds were prepared and diluted as indicated in either pyrogen free water or pyrogen free saline and tested in the LAL gel clot assay with a sensitivity of 0.01 ng/ml as determined by titration of standard LPS.

DRUG	ug/ml	LAL
AVS-1018	25	-
AVS-1761	0.1	+
AVS-1968	800	-
AVS-2149	0.4	+
AVS-2776	400	-
AVS-2777	400	-
AVS-2778	400	-
AVS-4286	80	+
AVS-4287	80	+
AVS-4593	80	+

Table 7. Effect of in vitro treatment with AVS-1018 on macrophage sensitivity to activating stimuli. Parallel cultures bone marrow culture derived macrophages were exposed to 25 μ g/ml of AVS-1018 in medium or medium alone for 24 h. The drug was then removed and activating stimuli added for 4 h. as indicated. After three washes to remove activators, the macrophage monolayers were cocultivated with ^{51}Cr -labelled, VEE-infected BALB/c3T3 cells for 16 h. Specific release was determined as described in Materials and Methods.

<u>TREATMENT</u>	<u>SPECIFIC RELEASE</u>	
LPS (ng/ml)	<u>Control</u>	<u>AVS-1018</u>
10	40	72
1	41	69
.01	45	72
.001	31	41
IFN		
gamma (5 U/ml)	44	44
+ 0.01 LPS	44	46
<i>a/B</i> (500 U/ml)	52	60
+ 0.01 LPS	44	66
Medium	37	28

Table 8. In vitro effects of drugs on macrophage activation. Bone marrow culture derived macrophages were cultured with medium or medium plus the indicated drugs at concentrations listed in Table 3. The macrophages were tested for their sensitivity to the activating stimuli listed. The results indicate changes relative to the reactivity of similarly activated medium treated macrophages. "+" increased reactivity, "0" no change from control reactivity, "-" decreased reactivity. If there was a difference in the response to α /BIFN and γ IFN, there are two listings in the IFN column. The first listing is for the response to α /BIFN and the second for γ IFN. A symbol in parentheses indicates a slight change in reactivity.

<u>DRUG</u>	<u>PERCENT SPECIFIC RELEASE</u>			
	<u>MED</u>	<u>LPS</u>	<u>IFN</u>	<u>IFN + LPS</u>
AVS-1018	0	+	0	(+)/0
AVS-1968	-	-	-	-
AVS-2776	+	+	+	+
AVS-2777	0	+	+	+
AVS-2778	-	-	-	-

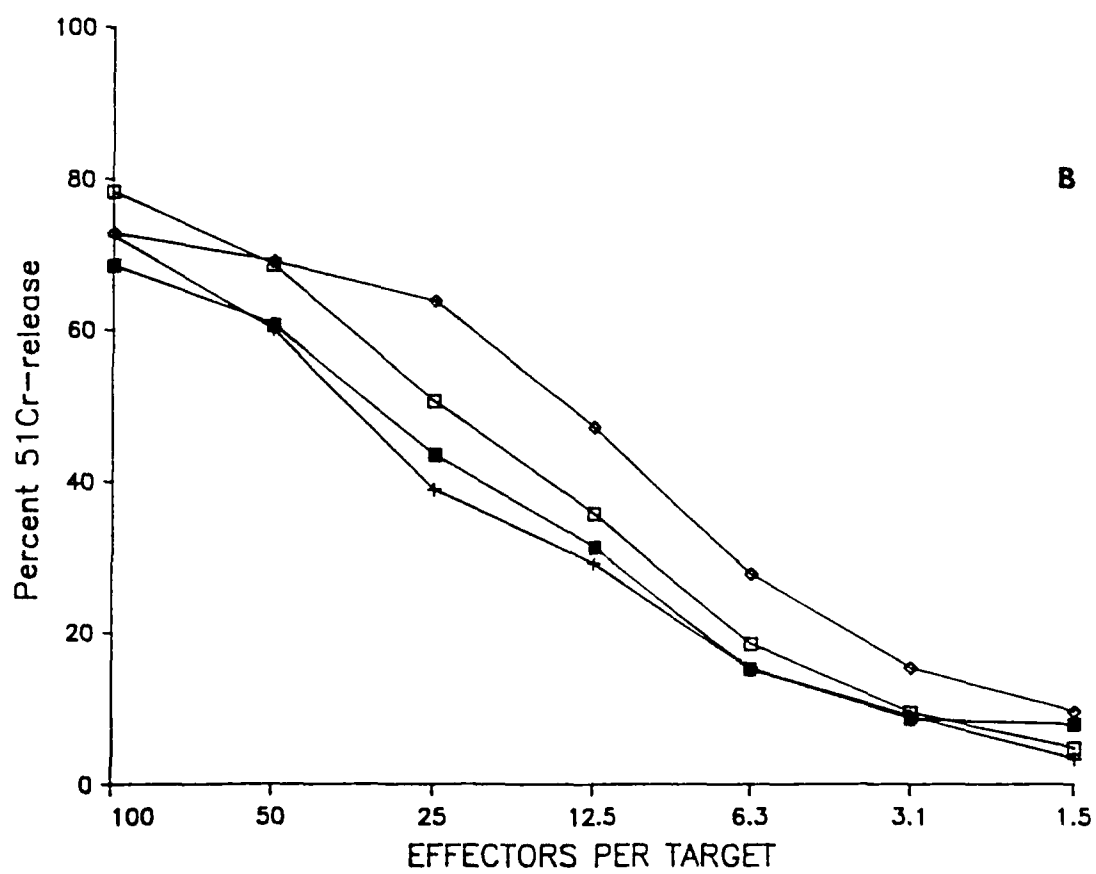
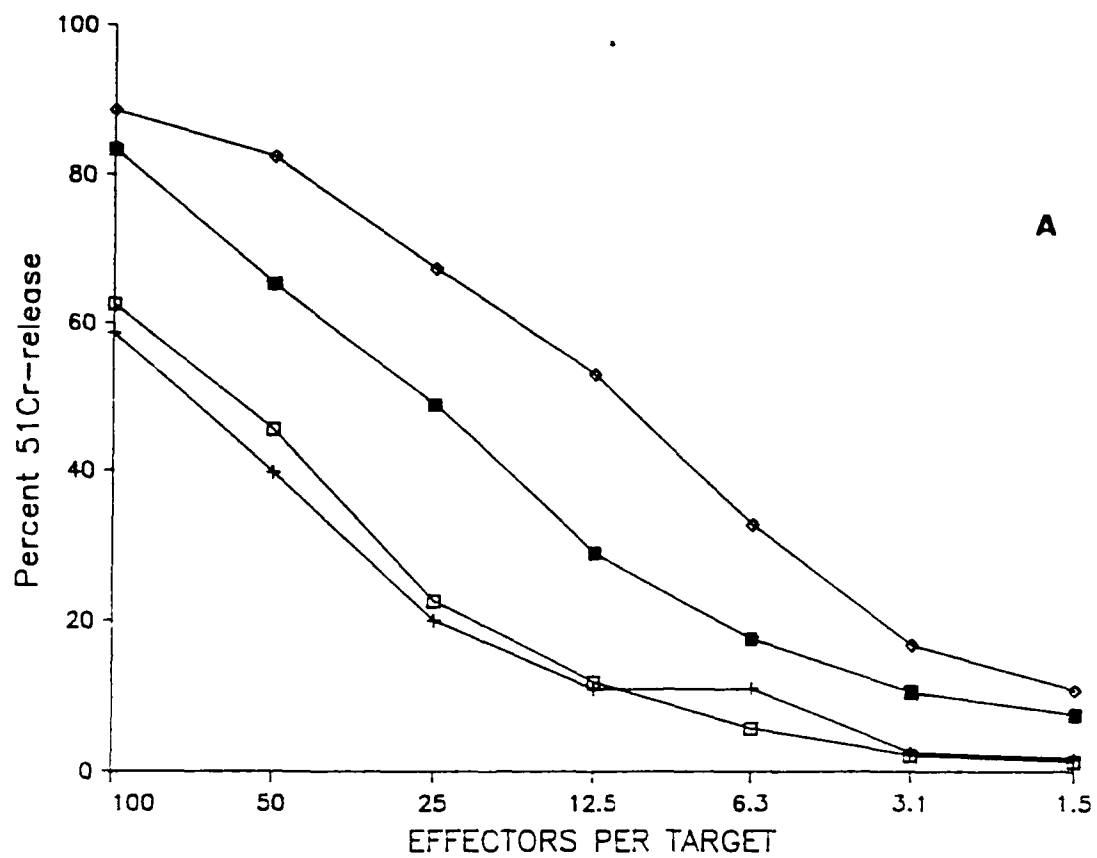
Table 9. In vivo effects of drugs on macrophage activation. Mice were treated with the indicated drug or control medium as for viral survival experiments (12,13). Peritoneal exudate cells were harvested when virus would have normally been given and macrophage monolayers prepared. The monolayers were tested for their sensitivity to the activating stimuli listed. The results indicate changes relative to the reactivity of similarly activated monolayers from control treated mice. "+" increased reactivity, "0" no change from control reactivity, "-" decreased reactivity. If there was a difference in the response to α /BIFN and γ IFN, there are two listings in the IFN column. The first listing is for the response to α /BIFN and the second for γ IFN. A symbol in parentheses indicates a slight change in reactivity.

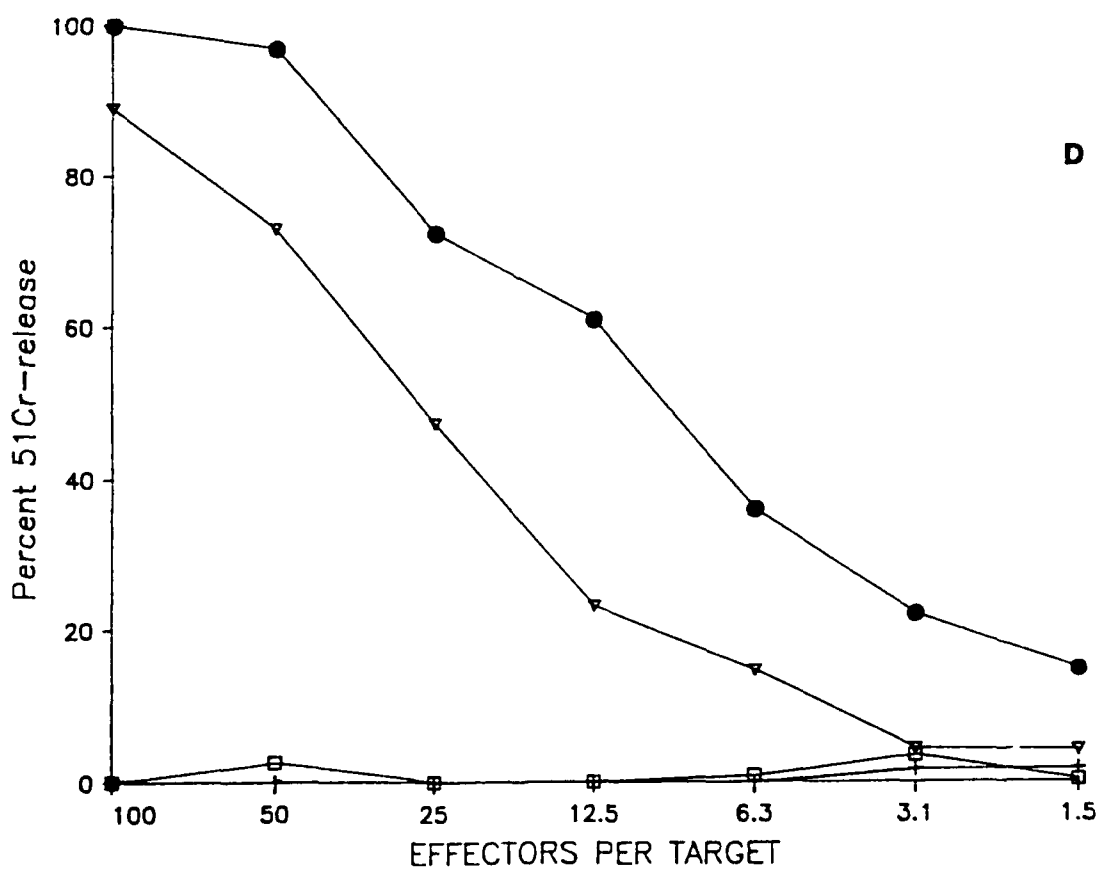
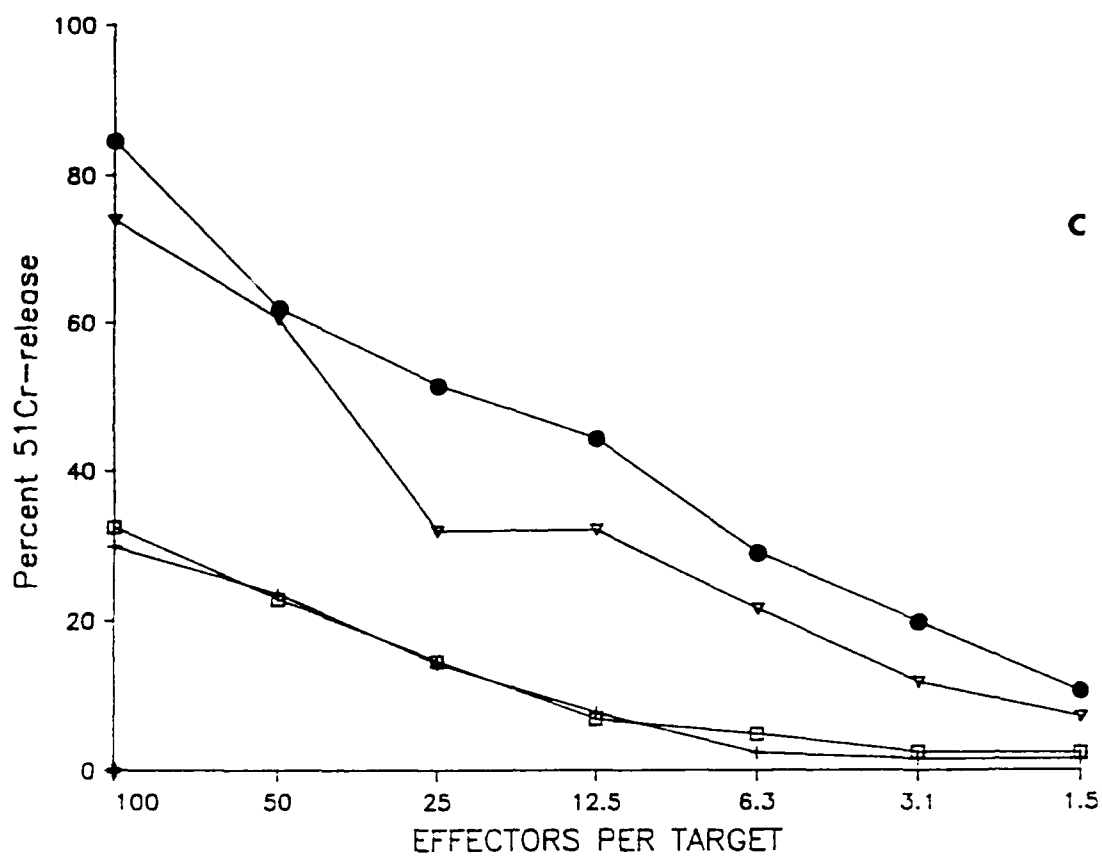
<u>DRUG</u>	<u>PERCENT SPECIFIC RELEASE</u>			
	<u>MED</u>	<u>LPS</u>	<u>IFN</u>	<u>IFN + LPS</u>
AVS-1018	0	(-)	(-)	0/(-)
AVS-1968	0	0	0	0/-
AVS-2776	0	+	0/+	+
AVS-2777	0	(+)	0	0
AVS-2778	-	0	-	0

Table 10. Changes in CTL activity induces by immunomodulator treatment. Mice were treated with the listed drugs or control medium as in survival experiments (12,13). At the time of virus challenge, mice were inoculated with either 100 pfu VEE, TC-83 strain, sc; or 2×10^6 F815 cells, ip. Eleven days later, the mice were sacrificed and their spleens removed. A single cell suspension was prepared from each pool of spleen cells and tested at multiple effector to target ratios against ^{51}Cr -labelled target cells. Those cells included a virus specific, histocompatible, VEE-infected L-929 cell, and the allogeneic F815 cell. The symbols indicate the change in activity against these targets of cells from drug treated mice as compared to control treated mice. "+" increased activity, "O" no change, "-" decrease in activity.

<u>DRUG</u>	<u>VIRUS</u>	<u>ALLOGENEIC</u>
1018	O	O
1761	-	O
1968	-	O
2149	+	-
2776	O	O
2777	O	O
2778	O	+
3960	O	O
4286	-	-
4287	O	-
4593	O	-

Figure 1. Effect of AVS 2149 treatment on development of antiviral and antiallogeneic CTL activity. Groups of 5 mice each were treated by intraperitoneal injection of either AVS-2149 (4 mg/kg) or PBS on day -1 with regard to antigen challenge. Mice were challenged by the subcutaneous injection of 10^3 pfu TC-83 passed once in chicken embryo cell culture in the presence of 4% chicken serum or the intraperitoneal injection of 1×10^6 P815 cells washed in serum-free medium to remove serum antigens. Control mice were left uninjected. Eleven days after antigen challenge, mice were sacrificed and each group used to prepare a pool of spleen cells: PBS alone (+), drug alone (\square), PBS plus P815 (\bullet), drug plus P815 (\blacktriangledown), PBS plus TC-83 (\blacksquare), drug plus TC-83 (\blacklozenge). These were tested at various concentrations against a constant number of ^{51}Cr -labelled target cells. The target cells were A) L929 infected with TC-83 at an moi of 3, B) L929 infected with VSV at an moi of 3, C) BALB/c3T3 infected with TC-83 at an moi of 3, and D) P815. Specific release was determined by the formula: Specific release = (experimental release - spontaneous release) / (maximum release - spontaneous release). Each value represents the mean of triplicate determinations.





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